PREVENTION OF INCORPORATION OF NON-STANDARD AMINO ACIDS INTO PROTEIN

[0001] This application claims the benefit of United States Provisional Application Serial Number 60/505,807, filed September 25, 2003.

FIELD OF THE INVENTION

[0002] The invention relates to the preparation of heterologous proteins from microorganisms and, more specifically, to preventing or substantially eliminating the incorporation of norleucine or other non-standard amino acids into these recombinant heterologous proteins. The present invention provides the compositions and methods necessary to prevent the incorporation of norleucine or other non-standard amino acids into these heterologous proteins.

BACKGROUND OF THE INVENTION

[0003] Norleucine is an analog of the amino acid methionine that can be misincorporated into a protein in the place of methionine. In *Escherichia coli* (*E. coli*) norleucine can be biosynthesized by the enzymes of the leucine biosynthetic pathway. When expressed in *E. coli* many heterologous proteins have norleucine mistakenly incorporated in places methionine residues should appear. The misincorporation of norleucine is undesirable because it usually results in the production of an altered protein, having less than optimal characteristics.

[0004] The amino acid norleucine (2-aminocaproic acid; 2-aminohexanoic acid; see Figure 1), first known to science from synthetic preparations made in 1870, attracted great interest after being claimed in 1882 by the chemist Ludwig Thudichum to have been found as one of the natural amino acids of proteins. Other workers seemed to confirm this finding, claiming in 1912–1913 to have found norleucine in proteins. These observations were ostensibly confirmed and extended by yet more laboratories during the following two decades. This body of literature was reviewed by Schmidt (1933), and led him to recommend that norleucine be added to the list of accepted constituent amino acids of proteins. However, within 12 years, it was conclusively shown that the analytical techniques employed by the earlier workers had misled them, and that norleucine did not naturally occur in proteins (Consden et al., 1945). The history of norleucine up to 1945, and the error in identifying it as a standard protein amino acid, is recounted in detail by Vickery (1972).

[0005] Prior to 1945, while norleucine was still considered to be a standard protein amino acid, nutritional studies with rats demonstrated that, rather than being an essential amino acid, norleucine was actually toxic (Rose, 1938). Norleucine was also shown to be toxic to *E. coli* and other species of bacteria. It was further observed that the growth inhibition of *E. coli* by norleucine was reversed by the addition of methionine to the growth medium, thereby establishing that norleucine is an analog of methionine (Harris and Kohn, 1941; Rowley, 1953; Adelberg, 1958; Rowbury, 1965; Karlstrom, 1965).

[0006] A review of these and other early reports that norleucine is inhibitory to a variety of species of bacteria is provided by Dittmer (1950). Moreover, Dittmer (1950) noted that norleucine is a structural analog of methionine by virtue of the fact that when the sulfur atom in methionine is replaced by a methylene group norleucine is the result (see Figure 1). Thus, norleucine was recognized to be an amino acid antagonist, and a structural analog, of methionine. Norleucine attracted significantly more interest than most amino acid analogs, since it was so well characterized and readily available—aspects stemming from the time when norleucine was thought to be a standard protein amino acid.

[0007] The first report of the incorporation of exogenously supplied norleucine into protein was that of Rabinowitz *et al.* in 1954, who observed that exogenous norleucine was incorporated into protein in rat Ehrlich ascites carcinoma cells. A similar observation was made a year later when it was shown that exogenous norleucine could be incorporated into casein in cows (Black and Kleiber, 1955).

[0008] These findings were followed, in 1956, by a demonstration that exogenous norleucine was also incorporated into protein by *E. coli* (Munier and Cohen, 1956). This observation was confirmed by later work (Nisman and Hirsch, 1958), and the phenomenon was also shown to occur in *Staphylococcus aureus* (Anfinson and Corley, 1969).

[0009] Shortly thereafter, it was shown that the incorporation of exogenous norleucine into *E. coli* protein occurred at the positions where methionine residues normally occurred in the proteins (Cohen and Munier, 1959; Munier and Cohen, 1959; Cowie et al., 1959). This discovery was also confirmed by later work (Neale and Tristam, 1963; Pine, 1967; Kerwar and Weissbach, 1970; Zipori, 1976). The early research into the use of norleucine as an analog of methionine, and its incorporation into protein (when supplied exogenously to a variety of

organisms) in place of methionine, was reviewed by Cohen and Gros (1963) and by Meister (1965).

[0010] By the mid-1960's it was widely known that exogenously supplied amino acid analogs that are incorporated into protein can have their incorporation blocked by the corresponding natural amino acid, especially when the natural amino acid is present in excess. The literature of that time provides several references establishing this general rule; including those found in Richmond (1962) and Fowden et al. (1967). Within a few years, it was appreciated that for an amino acid analog to be incorporated into protein it must compete with the naturally utilized amino acid for charging onto the corresponding tRNA (Pine, 1978, and Horton and Boime, 1983). These general rules for the incorporation of amino acid analogs into protein were highlighted by specific examples, including that the methionine analog norleucine was blocked from being incorporated into protein by the presence of methionine (Fowden et al., 1967; Pine, 1978; and Barker and Bruton, 1979).

[0011] Several studies independently demonstrated that the *E. coli* methionine-tRNA could be charged with norleucine *in vitro* and that this aberrant charging was inhibited by methionine (Trupin et al., 1966; Bruton and Hartley, 1968; Lemoine et al., 1968; Old and Jones, 1975; Old and Jones, 1977). Moreover, Old and Jones (1976) found that norleucine inhibited formation of methionyl-tRNA in an *E. coli* in vitro system; specifically, they showed that the level of methionine charging onto methionine-tRNA decreased gradually with increasing levels of norleucine.

[0012] *In vivo* studies also demonstrated that increased methionine pools reduced the incorporation of norleucine into protein. Fowden et al. (1967), in a review on amino acid analogs and their effects on *E. coli* and other organisms, stated (at page 91): "A general characteristic of all toxic analogs, whether synthetic or of natural origin, is that their toxic effects are specifically reversed by the normal protein amino acid which is antagonized by the analog", and (at page 92): "an analog, prior to incorporation into protein, must be activated and transferred to a specific transfer-RNA. The analog therefore must compete with the structurally related protein amino acid at the surface of an aminoacyl-tRNA synthetase". Fowler (at page 136), referring to the 1964 Ph.D. thesis of S. Neale (University of London), further stated that "the amount of norleucine incorporated into alkaline phosphatase of *E. coli* K-12 under

derepressed conditions was greatly reduced and the abnormally eluting enzyme was not apparent. Incorporation of the analog into the purified enzyme and into gross cell protein was decreased due to increased supplies of intracellular methionine".

[0013] Others have also demonstrated *in vivo* that low methionine levels typically produce relatively high norleucine incorporation. The level of norleucine incorporated into protein was increased in experiments employing mutants of *E. coli* unable to make their own methionine, especially when the methionine in the growth medium was exhausted (Yariv and Zipori, 1972; Naider et al., 1972; Brown, 1973). This same observation was made with *Staphylococcus aureus* (Anfinson and Corley, 1969). Brown (1973) used a mutant of *E. coli* unable to make its own methionine, grown in a medium containing a high ratio of norleucine to methionine, to prepare proteins with norleucine at the amino-terminus and at internal residues. Barker and Bruton (1979) studied norleucine incorporation into protein in *E. coli*, reporting in detail on the effects of different ratios of norleucine to methionine on the charging of methionine tRNA with norleucine, and to the subsequent incorporation of norleucine into protein. They demonstrated that the incorporation of norleucine into protein was dependent on the intracellular ratio of norleucine to methionine; significant incorporation of norleucine into protein occurred at a high ratio, and greatly reduced incorporation of norleucine into protein occurred at a low ratio.

[0014] It was clear to these workers, as discussed above, that norleucine was not a standard protein amino acid. Indeed, they concluded that norleucine did not even occur in nature as a free amino acid. However, this conclusion was disproved by the observation that *Serratia marcescens*, an organism closely related to *E. coli*, is able to biosynthesize norleucine when the leucine biosynthetic system is derepressed (Kisumi et al., 1976, 1977). In this organism, the enzymes of leucine biosynthesis were shown to be responsible for the biosynthesis of the endogenous norleucine. The leucine biosynthetic enzymes have broad substrate specificities (Bogosian et al., 1989), and are capable of forming both leucine and the structurally related norleucine (see Figure 1). These reports by Kisumi et al. (1976, 1977) represent the first observations of norleucine as a naturally occurring substance.

[0015] Thus, by the late 1970's, a great deal was understood about norleucine structure, use, and synthesis. It was clear that norleucine was a structural analog of methionine that could be incorporated into protein by mis-charged methionine-tRNA. Furthermore, it was clear that a

sufficient amount of available methionine inhibited the incorporation of norleucine into protein by out-competing norleucine for the charging of methionine-tRNA. Finally, it was known that norleucine was a naturally occurring amino acid, synthesized in bacteria by the enzymes of the leucine biosynthetic pathway.

[0016] The stage was thus set for a series of observations made by Bogosian and co-workers in 1985 and published a few years later (Bogosian *et al.*, 1989). They found that norleucine was undesirably incorporated into both native and heterologous proteins being expressed in recombinant strains of *E. coli*. The level of norleucine incorporation into these proteins ranged from 5% to 15% of the normal methionine content. In this case the norleucine was not being supplied exogenously, but was being naturally synthesized in the *E. coli* cells. They showed that, in *E. coli*, the enzymes of the leucine biosynthetic pathway also biosynthesized norleucine, and that the norleucine so formed could be incorporated into protein in place of methionine.

[0017] In an effort to produce heterologous proteins with a reduced norleucine content, Bogosian *et al.* went on to show that the incorporation of norleucine into protein could be reduced by adding additional methionine to the culture medium. They also showed that norleucine biosynthesis could be reduced by supplying exogenous leucine to the culture medium (thereby repressing the induction of leucine biosynthetic enzymes). It was also shown that inactivating one or more of the genes of the *leu* operon, which encodes the leucine biosynthetic enzymes, prevented the biosynthesis of norleucine (however, a bacterial strain unable to make its own leucine requires the addition of leucine to the culture medium).

[0018] Bogosian *et al.* also demonstrated that the initial substrate for norleucine biosynthesis was 2-ketobutryate, an intermediate in the biosynthesis of isoleucine. Thus, another approach employed by these workers to prevent the biosynthesis of norleucine was to inactivate the *ilvA* gene. The *ilvA* gene encodes threonine deaminase, the enzyme that initiates isoleucine biosynthesis by converting threonine to 2-ketobutyrate. However, the *ilvA* mutant was also incapable of making its own isoleucine. Consequently, this approach necessitated the addition of isoleucine to the culture medium. Thus, while a variety of approaches were devised by these workers to reduce the incorporation of norleucine into protein, they all required the addition of other amino acids (namely, methionine, leucine, or isoleucine) to the culture medium.

[0019] Other workers have made similar observations with other heterologous proteins expressed in recombinant *E. coli* strains. Norleucine was found to be incorporated into human interleukin-2 (Tsai et al. 1988, and Lu et al., 1988), recombinant human insulin-like growth factor I (Forsberg et al., 1990), human macrophage colony stimulating factor (Randhawa, 1994), human leptin (Liu et al., 1997), and human brain-derived neurotrophic factor (Sunasara et al., 1999). With these proteins, norleucine incorporation ranged from 5% to 20% of the normal methionine content.

[0020] Since norleucine is not a standard protein amino acid, it is desirable to minimize its incorporation into proteins in order to produce products that are as "natural" as possible (*i.e.* contain only the amino acids encoded by the DNA sequence). Previously devised methods for reducing the incorporation of norleucine into protein (Tsai et al. 1988, Bogosian et al., 1989, and Randhawa, 1994) were based on the prior art describing the biosynthesis of norleucine and the incorporation of norleucine into protein. That is, the prior art indicated that the biosynthesis of norleucine could be reduced by supplementation of the culture medium with leucine, thereby repressing the enzymes of leucine (and norleucine) biosynthesis. The art also indicated that inactivating the *ilvA* gene and/or one or more of the genes of the *leu* operon (namely *leuA*, *leuB*, *leuC*, and *leuD*) would reduce the biosynthesis of norleucine. Finally, the art indicated that supplementation of the culture medium with methionine would reduce the incorporation of norleucine into protein.

[0021] Thus, there are at least two approaches for preventing or reducing the incorporation of norleucine into heterologous proteins described in the existing art discussed above. (1) Inactivation of one or more of the genes encoding the biosynthetic enzymes necessary to produce norleucine. In *E. coli*, these genes include *ilvA*, *leuA*, *leuB*, *leuC*, and *leuD*. (2) Interference with the incorporation of norleucine into protein by supplementing the bacterial growth medium with methionine (or ALIMET® feed supplement, available from Novus International, Inc, St. Louis, Missouri, which *E. coli* can convert into methionine). That is, to competitively block norleucine incorporation into protein using this method, additional methionine accumulates inside the bacteria and competes with the available norleucine for attachment to the methionine tRNA, thereby reducing norleucine incorporation into protein.

[0022] Inactivation of one or more of the genes *leuA*, *leuB*, *leuC*, or *leuD* as a means of reducing norleucine incorporation into protein was also described by Fenton *et al.*, in U.S. Patent No. 5,599,690. Supplementation of the culture medium with methionine as a means of reducing norleucine incorporation into protein was also described by Fenton *et al.* in the '690 patent, and by Brunner *et al.*, in U.S. Pat. No. 5,698,418. Brunner *et al.*, in the '418 patent, also provide a description of a means for reducing norleucine incorporation into protein by supplementing the growth medium with other amino acids, specifically, leucine or cysteine. All of these approaches have the disadvantage of requiring the supplementation of the culture medium with one or more amino acids.

[0023] Another approach for preventing norleucine incorporation (also described by Brunner *et al.* in the '418 patent) is to mutate the protein-encoding gene at the codons originally encoding methionine so that they encode other amino acids. This approach has the disadvantage of altering the primary (and perhaps secondary and tertiary) structure of the protein, which may result in significant and undesirable changes in the biological properties, activity, and usefulness of the protein.

[0024] As discussed above, all approaches described, in the existing art, as being effective for reducing the incorporation of norleucine into protein, require either the supplementation of the culture medium with one or more amino acids or the mutation of the gene encoding the protein's amino acid sequence to eliminate methionine codons. It is desirable in the biotechnology industry to be able to cultivate recombinant organisms in a simple chemically defined minimal medium, without the need to add any expensive supplements, such as amino acids while simultaneously reducing the incorporation of norleucine into proteins. Furthermore, it is also desirable to do so without altering the protein's primary amino acid sequence.

[0025] Prior to the discovery of the invention disclosed in the instant application, there was no method known in the art that was able to achieve the objective of reducing the incorporation of norleucine into protein without requiring the supplementation of the culture medium with one or more amino acids and/or eliminating the methionine codons from the gene encoding the protein (thereby changing the protein's amino acid sequence).

[0026] Norvaline, another non-standard amino acid, is biosynthesized by the same pathway responsible for the synthesis of norleucine (*see*, Kisumi, *et al.* (1976) and Bogosian *et al.* (1989)).

[0027] Researchers have shown that, like norleucine, norvaline is sometimes inappropriately incorporated into heterologous proteins. For example, Chiu (1988) and Apostol *et al.* (1997) reported that norvaline can be incorporated into heterologous proteins, expressed in *Escherichia coli*, at positions normally occupied by leucine. Similarly Chiu (1988) and Kwong *et al.* (1998) reported that norvaline can be incorporated in heterologous proteins at positions normally occupied by methionine.

[0028] Additionally, other reports indicated that the non-standard amino acids beta-methylnorleucine (Muramatsu *et al.* (2002)) and homoisoleucine (Sunasara *et al.* (1999)) are sometimes inappropriately inserted into heterologous proteins, in the place of isoleucine.

[0029] Thus, there exists a need for methods of preventing or substantially reducing the incorporation of norleucine, norvaline, beta-methylnorleucine, homoisoleucine, and/or other non-standard amino acids into heterologous proteins. Such a method preferably would not require the use of expensive growth media or amino acid supplements. Neither should the method require alteration of the protein's amino acid sequence; instead the method should result in the incorporation of the proper amino acid into the protein.

PROBLEM SOLVED BY THE INVENTION

[0030] The instant invention meets this need for an efficient and inexpensive means of preventing the incorporation of norleucine and/or other non-standard amino acids into heterologous proteins. The instant invention meets this need by providing the methods and compositions necessary to prevent or substantially inhibit the incorporation of norleucine and/or other non-standard amino acids into heterologous proteins, without the necessity of supplementing the growth medium with amino acids or altering the protein's amino acid sequence to eliminate methionine or other naturally occurring amino acids.

[0031] The present invention meets this need by providing a method of reducing the incorporation of norleucine and/or other non-standard amino acids into proteins by degrading the norleucine and/or non-standard amino acids that the cell biosynthesizes. An important aspect of

this invention is that it provides a means for achieving a reduction or elimination of the incorporation of norleucine and/or other non-standard amino acids into proteins without necessitating the supplementation of the culture medium with any amino acids.

SUMMARY OF THE INVENTION

[0032] While there is extensive prior art on the degradation of amino acids (for example by a broad substrate enzyme such as a general amino acid oxidase), there is no suggestion in the existing art to using such an approach for reducing, or substantially eliminating, endogenous cellular levels of norleucine and/or other non-standard amino acids. Furthermore, there is no suggestion in the prior art describing such an approach for reducing endogenous cellular levels of norleucine and/or other non-standard amino acids by degradation for the ultimate purpose of reducing or substantially eliminating the incorporation of norleucine and/or other non-standard amino acids into proteins. In contrast, the instant invention provides for methods of reducing or preventing the incorporation of norleucine and/or other non-standard amino acids into proteins without having to supplement the growth medium with any amino acids or rich medium components.

[0033] Living organisms degrade excess amino acids to metabolic intermediates that can be used for other purposes. The major pathway of amino acid degradation starts with an oxidative deamination reaction that removes the alpha-amino group from the amino acid (Stryer, 1995). While little is known concerning the degradation of norleucine, or other non-standard amino acids, the few studies that have been conducted indicate that oxidative deamination is also the first step in the breakdown of norleucine and structurally related non-standard amino acids such as norvaline, beta-methylnorleucine, and homoisoleucine (see Figures 1 and 2). For example, oxidative deamination of norleucine would yield 2-ketocaproic acid (2-ketohexanoic acid; see Figure 1) and ammonia. Bender and Krebs (1950) observed oxidation of norleucine by amino acid oxidases of cobra venom and *Neurospora crassa*. Kinnory et al. (1955) reported that in rat liver homogenates norleucine degradation was by transamination and decarboxylation reactions, which yielded 2-ketocaproic acid, valeric acid, and beta-hydroxyvaleric acid. Greenberg (1961) reviewed this work and proposed a pathway by which norleucine was degraded first to

2-ketocaproic acid, which in turn was degraded to valeric acid and carbon dioxide, then to beta-ketovaleric acid, then to propionic acid and acetic acid.

[0034] The studies that have been published on the degradation of norleucine by bacteria suggest that this is an ability possessed by very few species of bacteria. Indeed, the degradation of norleucine by *Clostridium difficile* and *Peptostreptococcus anaerobius*, to the exclusion of other related species, is used as the basis of rapid identification tests for these pathogens (Nunez-Montiel et al., 1983; Turgeon et al., 1990).

[0035] While few studies have been published on the ability of bacteria to degrade norleucine *in vivo*, it is known from *in vitro* studies of several bacterial amino acid degradative enzymes that, in addition to their normal role in degrading standard protein amino acids, some of these enzymes also exhibit a low level ability to degrade norleucine.

[0036] For example, *in vitro* studies of phenylalanine dehydrogenase from *Thermoactinomyces intermedius* indicated that both the wild-type enzyme and a variant designated CS2 (with the substrate-binding domain of leucine dehydrogenase) were capable of degrading norleucine (via oxidative deamination) with 6% and 70%, respectively, of the activity against phenylalanine (Kataoka et al., 1993). Others have also reported that phenylalanine dehydrogenase from yet more species also degrades norleucine (see Table 1).

TABLE 1: Phenylalanine dehydrogenase enzymes showing activity against norleucine

Species	Activity against norleucine (as a percentage of activity against phenylalanine)	Reference
Bacillus badius	19	Asano *et al.* (1987)
Sporosarcina ureae	15	Asano *et al.* (1987)
Bacillus sphaericus	3.9	Asano *et al.* (1987)
Rhodococcus maris	16	Misano *et al.* (1989)
Thermoactinomyces intermedius	6.3	Kataoka *et al.* (1993)
Thermoactinomyces intermedius (CS2 mutant)*	65	Kataoka *et al.* (1993)

*A mutant with the substrate-binding domain of leucine dehydrogenase.

[0037] Furthermore, Turnbull et al. (1997), following up on the work of others, reported that *in vitro* studies showed that wild-type leucine dehydrogenase and valine dehydrogenase from

various species of bacteria (*e.g.*, *Streptomyces*, *Thermoactinomyces*, *Clostridium*, *Bacillus*, and *Corynebacterium*) were capable of degrading norleucine via oxidative deamination. See also Vancura *et al.* (1988) and Priestly and Robinson (1989), respectively reporting that norleucine is degraded by valine dehydrogenase from *Streptomyces fradiae* and *Streptomyces cinnamonensis*. Also Ohshima *et al.* (1994) reported that leucine dehydrogenase from *Thermoactinomyces intermedius* is active in norleucine degradation.

[0038] Many of the enzymes described above also exhibit activity against norvaline, in addition to their activity against norleucine (see Table 2). It would be expected that enzymes exhibiting activity against norleucine and/or norvaline would also exhibit activity against the structurally related non-standard amino acids beta-methylnorleucine and/or homoisoleucine (Figure 2 illustrates the structural similarities between these non-standard amino acids).

TABLE 2: Additional Enzymes showing activity against Norleucine and norvaline

Species	Enzyme	Activity against norleucine (as a percentage of activity against the indicated amino acid)	Activity against norvaline (as a percentage of activity against the indicated amino acid)	Reference
Bacillus badius	phenylalanine dehydrogenase	19 (phenylalanine)	5 (phenylalanine)	Asano *et al.* (1987)
Sporosarcina ureae	phenylalanine dehydrogenase	15 (phenylalanine)	6.3 (phenylalanine)	Asano *et al.* (1987)
Bacillus sphaericus	phenylalanine dehydrogenase	3.9 (phenylalanine)	1.3 (phenylalanine)	Asano *et al.* (1987)
Thermoactinomyces intermedius	phenylalanine dehydrogenase (wild-type)	6.3 (phenylalanine)	2.1 (phenylalanine)	Kataoka *et al.* (1993)
Thermoactinomyces intermedius	phenylalanine dehydrogenase (CS2 mutant)*	65 (phenylalanine)	36 (phenylalanine)	Kataoka *et al.* (1993)
Streptomyces fradiae	valine dehydrogenase	52 (Valine)	98 (Valine)	Vancura *et al. (1988)*
Streptomyces cinnamonensis	valine dehydrogenase	2.8 (valine)	26 (valine)	Priestley and Robinson (1989)
Streptomyces cinnamonensis	valine dehydrogenase	3 (valine)	26 (valine)	Turnbull *et al.* (1997)
Streptomyces aureofaciens	valine dehydrogenase	11 (valine)	43 (valine)	Turnbull *et al.* (1997)

Species	Enzyme	Activity against norleucine (as a percentage of activity against the indicated amino acid)	Activity against norvaline (as a percentage of activity against the indicated amino acid)	Reference
Streptomyces fradiae	valine dehydrogenase	52 (valine)	98 (valine)	Turnbull *et al.* (1997)
Alcaligenes faecalis	valine dehydrogenase	16 (valine)	44 (valine)	Turnbull *et al.* (1997)
Cornebacterium pseudodiptheriticum	leucine dehydrogenase	2 (leucine)	28 (leucine)	Turnbull *et al.* (1997)
Bacillus sphaericus	leucine dehydrogenase	10 (leucine)	41 (leucine)	Turnbull *et al.* (1997)
Bacillus licheniformis	leucine dehydrogenase	7 (leucine)	Not done	Turnbull *et al.* (1997)
Bacillus cereus	leucine dehydrogenase	6 (leucine)	28 (leucine)	Turnbull *et al.* (1997)
Thermoactinomyces intermedius	leucine dehydrogenase	3.6 (leucine)	27 (leucine)	Oshima *et al.* (1994)
Bos taurus (liver)	glutamate dehydrogenase	1.6 (glutamate)	17 (glutamate)	Struck and Sizer (1960)
Bos taurus (liver)	glutamate dehydrogenase	16 (glutamate)	100 (glutamate)	Tomkins *et al.* (1965)

*A mutant with the substrate-binding domain of leucine dehydrogenase.

[0039] Other enzymes that might degrade norleucine, norvaline, beta-methylnorleucine, and/or homoisoleucine (and/or other non-standard amino acids) include: other amino acid dehydrogenases, such as alanine dehydrogenase, glycine dehydrogenase, and opine dehydrogenase; aminotransferases (also known as transaminases); amino acid dehydratases; and various amino acid oxidases. It is noted that the list of enzymes, *supra*, especially those in Tables 1 and 2, is provided by way of example only, and is not exclusive. It would be well within the ability of those skilled in the art to identify related enzymes from the same or other species and employ these enzymes in accordance with the instant invention. Thus, the enzymes contemplated as being within the scope of the current invention reaches beyond those listed in Tables 1 and 2 (for example, enzymes contemplated as being part of the instant invention also includes, but is not limited by, those enzymes listed in Table 4, *infra*).

[0040] Given the similarity in structure between the non-standard amino acids and the standard amino acids (see Figure 2), it is believed that the mechanism for the metabolism of the non-standard amino acids (including norleucine), by the various enzymes listed herein, is analogous to that the mechanism used to metabolize the normal substrate of such enzymes. Moreover, given the structural similarity among the super-family of enzymes that includes, at a minimum, glutamate dehydrogenases, leucine dehydrogenases, phenylalanine dehydrogenases, valine dehydrogenases, glutamate/leucine/phenylalanine/valine dehydrogenases, and opine dehydrogenases (the latter being *e.g.* from *Arthrobacter sp.*), it is likely that all of these enzymes will have at least some activity against norleucine, norvaline, homoisoleucine, beta-methylnorleucine and other non-standard amino acids.

[0041] The instant invention provides for methods for preparing recombinant strains of bacteria (*e.g.*, *E. coli*) with co-expression or enhanced expression of glutamate dehydrogenases, leucine dehydrogenases, phenylalanine dehydrogenases, valine dehydrogenases, glutamate/leucine/phenylalanine/valine dehydrogenases, opine dehydrogenases, other amino acid dehydrogenases, and other enzymes such as aminotransferases (also known as transaminases), amino acid dehydratases, and various amino acid oxidases, exhibiting activity for the degradation of norleucine and/or other non-standard amino acids, including norvaline, homoisoleucine, and beta-methylnorleucine. In addition, the instant invention provides for variants of these enzymes exhibiting increased activity for the degradation of norleucine and/or other non-standard amino acids, including norvaline, homoisoleucine, and beta-methylnorleucine.

[0042] One example of an enzyme exhibiting activity for the degradation of norleucine, and for which variants are known exhibiting increased activity for the degradation of norleucine, is glutamate dehydrogenase. Glutamate dehydrogenase (GDH) is an enzyme that degrades the amino acid glutamate via oxidative deamination to form 2-ketoglutarate and ammonia (see Figure 1). GDH from the organism *Clostridium symbiosum* has been crystallized and studied extensively. A variant form of the *Clostridium symbiosum* GDH has been identified, in which the lysine residue at position 89 has been changed to a leucine residue (this is referred to as the K89L form of GDH). This GDH variant exhibits an increased ability to degrade norleucine (Stillman et al., 1999; Wang et al., 2001; Goyal et al., 2001).

[0043] The present invention provides for glutamate dehydrogenase (GDH) from *E. coli* (both wild-type GDH and variants comprising a lysine 92 to leucine, K92L, variation of *E. coli* GDH; the lysine residue that is at position 89 in the *Clostridium symbiosum* GDH is at position 92 in the *E. coli* GDH) that efficiently degrades norleucine. That is, the instant invention provides for recombinant DNA molecules encoding the GDH proteins described as well as the recombinant proteins encoded. The instant invention also provides for methods for preparing recombinant strains of bacteria (*e.g.*, *E. coli*) with enhanced expression of the wild-type GDH gene and/or enhanced expression of the K92L variant form of *E. coli* GDH. The instant invention also provides for methods for preparing recombinant strains of bacteria (*e.g.*, *E. coli*) with co-expression or enhanced expression of leucine dehydrogenases, valine dehydrogenases, and glutamate/leucine/phenylalanine/valine dehydrogenases. In any embodiment of the instant invention, the modified cell has co-expression or enhanced expression of the norleucine degrading enzyme as compared with its expression in the unmodified cell. Various embodiments of the instant invention provide new protein expression systems in which heterologous proteins can be produced, where these proteins have a reduced or substantially eliminated norleucine content, and yet the bacteria are grown on a minimal medium; and, thus, do not require supplementation with any amino acids whatsoever (nevertheless, supplemental amino acids may be added). Also provided are the bacterial strains so produced.

[0044] The instant invention also provides various means for reducing the incorporation of norleucine and/or other non-standard amino acids into heterologous proteins without the use of expensive amino acid supplements. That is, the methods of the instant invention do not require provision of exogenous amino acids (such as leucine, methionine, valine, or isoleucine) to compensate for the inhibition of a amino acid biosynthetic pathway, nor excessive methionine required in order to competitively inhibit the incorporation of norleucine or other non-standard amino acids into proteins.

[0045] Notwithstanding that the instantly claimed invention effectively reduces or eliminates the incorporation of norleucine and/or other non-standard amino acids into native or heterologous proteins without the addition of amino acids supplements, various aspects of the instant invention also provide for the use of one or more amino acid supplements in combination with cells having co-expression or enhanced expression of one or more proteins capable of degrading norleucine

and/or one or more other non-standard amino acids. By this means, it is possible to even further reduce the incorporation of norleucine and/or other non-standard amino acids into heterologous proteins (at least in those instances where non-standard amino acid content is not already substantially zero).

[0046] The instant invention provides methods and compositions that prevent or substantially eliminate the incorporation of norleucine and/or other non-standard amino acids into heterologous proteins by engineering a cell so that it degrades most or all of the norleucine, and/or other non-standard amino acids, that it synthesizes.

[0047] According to various embodiments of the invention, the prevention of the incorporation of norleucine and/or other non-standard amino acids into a heterologous protein is accomplished by co-expressing the heterologous protein in a cell with co-expression or enhanced expression of a protein, or enzymatically functional portion of a protein, that degrades norleucine and/or other non-standard amino acids. The various aspects of this embodiment provide for a microorganism co-expressing at least one heterologous protein and at least one non-standard amino acid degrading protein (or enzymatically active portion thereof).

[0048] As indicated above, other embodiments of the invention provide for recombinant DNA molecules capable of encoding an enzyme that degrades norleucine and/or other non-standard amino acids, or recombinant proteins capable of degrading norleucine and/or other non-standard amino acids.

[0049] Other embodiments of the instant invention provide for methods of purifying heterologous proteins having a reduced content of norleucine and/or other non-standard amino acids.

DESCRIPTION OF THE FIGURES

[0050] The following figure forms part of the present specification and is included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to this figure in combination with the detailed description of specific embodiments presented herein.

[0051] **Figure 1:** Shows the basic structures for the indicated amino acids (methionine, norleucine, leucine, and glutamate). Also shown are the results of oxidative deamination of norleucine and glutamate, respectively.

[0052] **Figure 2A-2B:** Show the structures for the indicated amino acids: methionine, norleucine, norvaline, and leucine (Fig. 2A); homoisoleucine, isoleucine, valine, and beta-methylnorleucine (Fig. 2B).

DESCRIPTION OF THE SEQUENCE LISTINGS

[0053] The following sequence listings form part of the present specification and are included to further demonstrate certain aspects of the present invention. The invention may be better understood by reference to one or more of these sequences in combination with the detailed description of specific embodiments presented herein.

SEQ ID NO:	Description
1	DNA sequence of wild-type *E. coli* glutamate dehydrogenase
2	Protein sequence of the wild-type *E. coli* glutamate dehydrogenase.
3	DNA sequence encoding the *E. coli* K92L glutamate dehydrogenase variant.
4	Protein sequence of the *E. coli* K92L glutamate dehydrogenase variant.
5	DNA sequence of *Bacillus cereus* leucine dehydrogenase
6	Protein sequence of *Bacillus cereus* leucine dehydrogenase.
7	DNA sequence of *Bacillus subtilis* leucine dehydrogenase.
8	Protein sequence of *Bacillus subtilis* leucine dehydrogenase.
9	DNA sequence of *Nostoc sp.* leucine dehydrogenase.
10	Protein sequence of *Nostoc sp.* leucine dehydrogenase.
11	DNA sequence of *Shewanella oneidensis* leucine dehydrogenase.
12	Protein sequence of *Shewanella oneidensis* leucine dehydrogenase.
13	DNA sequence of *Streptomyces avermitilis* valine dehydrogenase.
14	Protein sequence of *Streptomyces avermitilis* valine dehydrogenase.
15	DNA sequence of *Nitrosomonas europaea* glutamate/ leucine/phenylalanine/valine dehydrogenase.
16	Protein sequence of *Nitrosomonas europaea* glutamate/ leucine/phenylalanine/valine dehydrogenase.

DEFINITIONS

[0054] The following definitions are provided in order to aid those skilled in the art in understanding the detailed description of the present invention.

[0055] As used herein, the term "heterologous protein(s)" preferably refers to a protein that is not expressed in the organism in an untransformed state. Put another way, it means that the protein is not native to the organism. As used herein the term "heterologous protein" does not encompass any protein that is typically or routinely used as a "marker" (meaning a selection marker). Such "markers" include, but are not limited to antibiotic resistance genes and proteins capable of processing substrate so as to provide a colored product for a colorimetric assay.

[0056] As used herein the terms "co-express", "co-expresses", and "co-expressed" refer to proteins/DNA molecules which are expressed in a cell as a result of a recombinant event. That is, at least one of the following is true: either the DNA and/or protein is expressed from an extra genomic vector (such as a plasmid) that has been introduced into the cell via a molecular biological technique; and/or the DNA/protein is expressed from a location in the cell's genome other than where the DNA sequence naturally occurs.

[0057] As used herein the term "co-expression" or "enhanced expression" refers to the modification of a cell so that the expression of a particular RNA transcript or protein is increased in that modified cell as compared with the level of expression of that same RNA or protein in an unmodified cell. Means for co-expression or enhanced expression contemplated as being part of the instant invention include, but are not limited to: expression of the gene from an extra-genomic DNA molecule (*e.g.* a plasmid); expression of the gene from a non-native location in the cellular genome; and/or expression of the gene from its native genomic location, but with modification of the gene's normal regulatory control system so as to stimulate expression or reduce suppression (that is any modification which increases the gene's expression).

[0058] Thus, as used herein the terms "co-expressing", "co-expression", and "enhanced expression" refers to at least two distinct phenomena. One aspect of co-expression or enhanced expression is the increased expression of a gene sequence already present in the cell (*e.g.* the increased expression, in *E. coli*, of RNA and/or protein that is native to *E. coli*, such as *E. coli* glutamate dehydrogenase) so that the RNA and/or protein encoded by the native sequence is present at higher levels than in the non-modified cell. A second aspect of co-expression or

enhanced expression is the expression of a "new" sequence that is not native to the cell. This would include, for example, the expression of the K92L glutamate dehydrogenase variant in an *E. coli* strain that did not previously produce the messenger RNA or protein for the K92L variant of glutamate dehydrogenase. In sum, "co-expression" or "enhanced expression" refers to both the "increased" expression of a native RNA and/or protein, and the "new" expression of a non-native RNA and/or protein. Thus, as used herein all variants of the terms "co-expression" and "enhanced expression" denote expression of an RNA and/or protein in a microorganism at a level that is greater than the level, of that same RNA and/or protein, expressed by the same microorganism in its unmodified form (*i.e.* a microorganism that is not "co-expressing" the RNA and/or protein).

[0059] As used herein the terms "norleucine and/or non-standard amino acid degrading enzyme" and "non-standard amino acid degrading protein" preferably refer to enzymes and/or proteins, or catalytically active fragments thereof, that degrade one or more of the non-standard amino acids; these nonstandard amino acids including, but not limited to: norleucine, norvaline, beta-methylnorleucine, and homoisoleucine. Non-standard amino acid degrading proteins include, but are not limited to, all of those proteins specifically described herein (and/or listed in any of the tables herein) as being capable of degrading one or more non-standard amino acids. They also include, but are not limited to, proteins structurally related to those specifically described proteins (*e.g.* see Table 4). Such proteins include the protein super-family comprising: glutamate dehydrogenases, leucine dehydrogenases, valine dehydrogenases, phenylalanine dehydrogenases, glutamate/leucine/phenylalanine/valine dehydrogenases, and opine dehydrogenases.

[0060] As used herein the term "non-standard amino acids" preferably refers to one or more amino acids that are not among the 20 amino acids most commonly found in proteins produced by living organisms. For the purposes of the instant invention, the "standard" amino acids are: 1) alanine, 2) arginine 3) asparagine, 4) aspartate, 5) cysteine, 6) glutamate, 7) glutamine, 8) glycine, 9) histidine, 10) isoleucine, 11) leucine, 12) lysine, 13) methionine, 14) phenylalanine, 15), proline, 16) serine, 17) threonine, 18) tryptophan, 19) tyrosine, and 20) valine. Non-standard amino acids include, but are not limited to, norleucine, norvaline, beta-methylnorleucine, and homoisoleucine.

[0061] As used herein the term "substantially eliminates" as it pertains to the presence of norleucine or other non-standard amino acids in proteins preferably means that there is no norleucine or other non-standard amino acids present in the proteins or that their presence is so low that it is below the limits of detection.

DETAILED DESCRIPTION OF THE INVENTION

[0062] The instant invention provides for compositions and methods useful to prevent or substantially eliminate the incorporation of one or more non-standard amino acids (including, but not limited to: norleucine, norvaline, beta-methylnorleucine, and/or homoisoleucine) into heterologous proteins. Various embodiments of the instant invention provide for methods that prevent incorporation of norleucine, norvaline and/or the other non-standard amino acids into proteins that are heterologously expressed. In certain aspects of this embodiment of the invention the incorporation of norleucine and/or other non-standard amino acids into heterologous proteins is prevented or substantially eliminated by co-expression of the heterologous protein in a cell with co-expression or enhanced expression of at least one enzyme/protein (or a catalytically active fraction thereof) that catalyzes the degradation of norleucine and/or one or more other non-standard amino acids. That is, the instant invention provide for microorganisms co-expressing at least one heterologous protein and at least one non-standard amino acid degrading protein.

[0063] In one aspect of this embodiment the norleucine or other non-standard amino acid degrading protein is a glutamate dehydrogenase (GDH). In a particularly preferred aspect of this embodiment the norleucine or other non-standard amino acid degrading enzyme is GDH from *Escherichia coli* (*E. coli*). In another preferred aspect of this embodiment the norleucine or other non-standard amino acid degrading protein comprises a lysine 92 to leucine (K92L) variant of *E. coli* GDH. In a particularly preferred embodiment of the invention the heterologous protein is co-expressed in *E. coli* with enhanced expression of either an native *E. coli* GDH (or a enzymatically active fragment thereof) or a norleucine degrading protein comprising a K92L variant of *E. coli* GDH (or an enzymatically active fragment thereof). In another preferred aspect of this embodiment the norleucine or other non-standard amino acid degrading protein comprises a leucine dehydrogenase, or a valine dehydrogenase, or a glutamate/ leucine/phenylalanine/valine dehydrogenase. In any aspect of the current invention it is

contemplated that the modified cell has co-expression or enhanced expression of the norleucine or other non-standard amino acid degrading protein as compared with the protein's expression in the non-modified cell. In other aspects of the present invention the GDH K92L variant may further comprise other variations from the native sequence. All such variants are considered to be part of the instant invention so long as they do not diminish the protein's ability to degrade norleucine or other non-standard amino acids to a degree where it is no longer useful according to the instant invention.

[0064] In other aspects of this embodiment of the invention the non-standard amino acid degrading protein may be selected from any protein found to produce a suitable degree of degradation of norleucine and/or other non-standard amino acids. Thus, in addition to glutamate dehydrogenase, other proteins provided for use according to the instant invention include, but are not limited to, phenylalanine dehydrogenase (examples of such a phenylalanine dehydrogenases are shown in Tables 1 and 2, *supra*, and Table 4, *infra*. These include both wild-type and variant enzymes isolated from *Thermoactinomyces intermedius*, but this is not an exclusive list), leucine dehydrogenase, valine dehydrogenase (exemplary leucine and valine dehydrogenases include, but are not limited to those obtained from *Streptomyces*, *Thermoactinomyces*, *Clostridium*, *Bacillus*, and *Corynebacterium*, see also the examples listed in Tables 1 and 2, *supra*), and other amino acid dehydrogenases, such as glutamate/leucine/phenylalanine/valine dehydrogenase, alanine dehydrogenase, glycine dehydrogenase, and opine dehydrogenase; aminotransferases (also known as transaminases); amino acid dehydratases; and various amino acid oxidases. More preferably, the non-standard amino acid degrading enzymes are selected from the group consisting of: glutamate dehydrogenases, leucine dehydrogenases, valine dehydrogenases glutamate/leucine/phenylalanine/valine dehydrogenases, phenylalanine dehydrogenases, and opine dehydrogenases.

[0065] Thus, in various embodiments of the invention the non-standard amino acid to be degraded is selected from one or more of the group consisting of norleucine, norvaline, beta-methylnorleucine, and homoisoleucine and the non-standard amino acid degrading enzyme is selected from one or more of the following: a glutamate dehydrogenase, a phenylalanine dehydrogenase, a leucine dehydrogenase, a valine dehydrogenase, a glutamate/

leucine/phenylalanine/valine dehydrogenase and an opine dehydrogenase (nevertheless these lists are not exclusive).

[0066] In one aspect of this embodiment of the instant invention the non-standard amino acid degrading enzyme, degrades norleucine and/or other non-standard amino acids and is encoded by a DNA molecule comprising a sequence as provided in SEQ ID NO:1, 3, 5, 7, 9, 11, 13, or 15. In another aspect of this embodiment of the invention the norleucine and/or other non-standard amino acid degrading enzyme has a peptide sequence comprising the sequence of SEQ ID NO:2, 4, 6, 8, 10, 12, 14, or16.

[0067] In various embodiments of the invention the heterologous protein is any protein or protein fragment of interest that can be advantageously expressed in bacteria. In certain preferred aspects of this embodiment of the invention the heterologous protein is a somatotropin. In more preferred aspects of this embodiment the somatotropin is a human, bovine, equine, porcine, ovine, canine, or feline somatotropin. In a particularly preferred aspect of this embodiment the heterologous protein is bovine somatotropin (bST).

[0068] Other heterologous proteins to which the instant invention is drawn include, but are not limited to human interleukin-2, recombinant human insulin-like growth factor, human growth factor, human macrophage colony stimulating factor (M-CSF), human leptin, and human brain-derived neurotrophic factor. These proteins are exemplary only, the list is not exclusive. Accordingly, any heterologous protein for which the exclusion of norleucine and/or one or more other non-standard amino acids is desired or necessary, may advantageously be produced in accordance with the instantly described invention.

[0069] Other embodiments of the instant invention provide for the exclusion of certain "marker" polypeptides from the list of those "heterologous" proteins that are envisioned as being advantageously co-expressed with the norleucine and/or other non-standard amino acid degrading protein.

[0070] Proteins that are contemplated as being part of this group of "marker peptides" include all proteins commonly used by those of ordinary skill in the art as a means of identifying cells that have been transformed. This list includes, but is not limited to antibiotic resistance genes such as ampicillin resistance genes, chloramphenicol acetyl transferase (CAT), tetracycline resistance, kanamycin resistance, neomycin resistance, streptomycin resistance, spectinomycin resistance,

gentamicin resistance, and zeocin resistance. This list also includes proteins that are essential for the maintenance of the plasmid, such as proteins involved in plasmid DNA replication, regulation of plasmid copy number, and plasmid mobilization and transfer. This list also includes proteins used to select for the presence of plasmid inserts, such as positive selection markers.

[0071] Other embodiments of the instant invention provide for purification of the co-expressed heterologous protein for advantageous use elsewhere. For example, in one aspect of this embodiment of the invention the heterologous protein is a bovine somatotropin that is to be isolated for use in cattle or another susceptible animal. It is typically important that a heterologous peptide be of its native sequence (or as close thereto as possible) when it is to be used in a higher organism, such as a mammal. For these uses, proteins having minimal norleucine and/or other non-standard amino acid content are most desirable. Similarly, for this reason it is also desirable to express proteins having their native sequence (*i.e.* not mutated to replace codons for methionine or other standard amino acids with codons encoding a different amino acid, in an effort to prevent incorporation of norleucine and/or other non-standard amino acids).

[0072] Various embodiments of the invention provide for the co-expression of any desired heterologous protein in a cell with co-expression or enhanced expression of one or more norleucine and/or other non-standard amino acids degrading proteins (or enzymatically active fragments thereof). For example, bovine somatotropin (bST) or any other type of somatotropin (ST) can be co-expressed in a cell with enhanced expression of wild-type *E. coli* GDH (or with enhanced expression of the K92L *E. coli* GDH variant). Alternatively, a desired heterologous protein can be co-expressed in a cell modified to have co-expression or enhanced expression of any other norleucine and/or other non-standard amino acid degrading protein or a catalytically active fragment of any such protein.

[0073] Accordingly, one particularly preferred embodiment of the instant invention provides for bST, or another somatotropin, being co-expressed in *E. coli* with enhanced expression of *E. coli* GDH or enhanced expression of a K92L variant of *E. coli* GDH. According to various aspects of this embodiment of the invention, the *E. coli* strain may be a K-12 strain or any other strain suitable for protein expression.

[0074] Nevertheless, the methods of the instant invention may be carried out using any desired combination of norleucine and/or other non-standard amino acids degrading protein, heterologous protein, and host cell. That is, the invention is not limited to any particular combinations of cell, norleucine and/or other non-standard amino acids degrading protein, and heterologous protein. Rather, all possible combinations and/or permutations of the cells, norleucine and/or other non-standard amino acids degrading proteins, and heterologous proteins described herein are envisioned as being part of the instant invention.

[0075] Various embodiments of the instant invention also provide for methods of producing and/or isolating proteins wherein the percent of proteins comprising norleucine and/or other non-standard amino acids has been reduced by at least 50% (as compared with the level of heterologous protein comprising norleucine and/or other non-standard amino acid(s), when the heterologous protein is produced in the same cell type and under the same conditions, except that the cell does not have co-expression or enhanced expression of a norleucine and/or other non-standard amino acid degrading protein). More preferably, the percent reduction in norleucine and/or or other non-standard amino acid content is 60%, 70%, 80%, 90%, 95, 96, 97, 98, 99, or greater than 99%, and includes substantially 100% (*i.e.*, no detectable non-standard amino acid). That is, in any embodiment of the invention, the percentage of heterologous protein comprising norleucine, and/or one or more other non-standard amino acids, is substantially zero.

[0076] The percent reduction in norleucine (and/or other non-standard amino acid) content is typically calculated as a reduction in percentage of proteins containing norleucine (and/or other non-standard amino acid). Nevertheless, any suitable method for analyzing the reduction in norleucine (and/or other non-standard amino acid) content may be used, such as calculating the amount of norleucine (and/or other non-standard amino acid) present in heterologous proteins isolated from cells that do not have co-expression or enhanced expression of a norleucine (and/or other non-standard amino acid) degrading protein and then comparing this result with the amount of norleucine (and/or other non-standard amino acid) in heterologous proteins present in heterologous proteins isolated from cells grown under identical conditions, except that the cells have co-expression or enhanced expression of a norleucine (and/or other non-standard amino acid) degrading protein.

[0077] Other embodiments of the instant invention provide for methods of producing cells that have co-expression or enhanced expression of a norleucine (and/or other non-standard amino acid) degrading protein wherein the cells have a decreased pool of norleucine (and/or other non-standard amino acids), as compared with the same cells that do not express the norleucine (and/or other non-standard amino acid) degrading protein, when grown under conditions that are suitable to elicit norleucine (and/or other non-standard amino acid) production. In preferred aspects of this embodiment of the invention, the amount of norleucine and/or other non-standard amino acids present in the cells' amino acid pool is decreased by at least 20%. In more preferred aspects of this embodiment the amount of norleucine and/or other non-standard amino acids present in the amino acid pools of the cells is decreased by 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98%, 99%, greater than 99% or substantially 100%.

[0078] Another method of measuring the reduction in amount of norleucine present is as a function of an increased ratio of methionine to norleucine (or, more generally, the ratio of the standard amino acid to the non-standard amino acid that can replace it, examples include, but are not limited to: the ratio of leucine to norvaline or methionine to norvaline and the ratio of isoleucine to homoisoleucine or isoleucine to homoisoleucine). In various aspects of this embodiment of the invention the methionine to norleucine (or standard amino acid to non-standard amino acid) ratio is preferably increased to at least 1.2:1, more preferably the ratio is increased to 1.3:1, 1.4:1, 1.5:1, 1.6:1, 1.7:1, 1.8:1, or 1.9:1. More preferably the ratio is at least 2.0:1. Even more preferably, the ratio is greater than 2.0:1.

[0079] The cell may be of any type suitable for expression of a heterologous protein with simultaneously co-expression or enhanced expression of a norleucine (and/or other non-standard amino acid) degrading protein. In a preferred aspect of this embodiment the cell is from an organism that synthesizes norleucine (and/or one or more other non-standard amino acids) and incorporates such into heterologous protein. In a more preferred embodiment of this aspect of the invention, the cell expresses the norleucine (and/or other non-standard amino acid) degrading protein at a higher rate than the norleucine (and/or other non-standard amino acid) degrading protein is expressed in the native (non-transformed) cell. In an even more preferred embodiment, the cell is an *E. coli* cell.

[0080] In various aspects of the embodiments described above the reduction in the content of the norleucine (and/or other non-standard amino acids) in heterologous proteins or reduction in norleucine (and/or other non-standard amino acids) content in the amino acid pool of the cell is accomplished by the co-expression or enhanced expression of one or more norleucine (and/or other non-standard amino acid) degrading proteins in the cell, in accordance with the methods described herein. Such co-expression or enhanced expression may be from an extra-genomic vector such as a plasmid or it may be from a genomic sequence that is not native to the cell, including expression from a non-native gene that has been integrated into the chromosome of the host cell, or it may result from a modification of the norleucine (and/or other non-standard amino acid) degrading protein's native regulatory control mechanism.

[0081] As described, the present invention envisions that the various aspects of the invention may be used in any combination with any of the other aspects described herein. Accordingly, the aspects of this embodiment of the invention include the co-expression of any heterologous protein with any suitable norleucine (and/or other non-standard amino acid) degrading protein in any suitable cell type. Nevertheless, by way of non-exclusive example, it is noted that preferred embodiments of the invention are drawn to the co-expression of heterologous proteins in a cell with co-expression or enhanced expression of a norleucine (and/or other non-standard amino acid) degrading protein selected from one or more of the following: a glutamate dehydrogenase, a phenylalanine dehydrogenase, a valine dehydrogenase, a leucine dehydrogenase, a glutamate/leucine/phenylalanine/valine dehydrogenase, and an opine dehydrogenase; other amino acid dehydrogenases, such as alanine dehydrogenase and glycine dehydrogenase; aminotransferases (also known as transaminases); amino acid dehydratases; and various amino acid oxidases. Also contemplated by the instant invention is the use of catalytically active fragments or catalytically active variants of any of the foregoing.

[0082] In particularly preferred embodiments of this aspect of the invention the norleucine (and/or other non-standard amino acid) degrading protein is a glutamate dehydrogenase, a leucine dehydrogenase, a valine dehydrogenase, or a glutamate/leucine/phenylalanine/valine dehydrogenase. In an even more preferred aspect the norleucine (and/or other non-standard amino acid) degrading protein is *E. coli* glutamate dehydrogenase or a lysine 92 leucine variant of *E. coli* glutamate dehydrogenase. In an even more preferred aspect of this embodiment the

glutamate dehydrogenase comprises the amino acid sequence of SEQ IN NO:2 or SEQ ID NO:4. More preferably, the glutamate dehydrogenase is encoded by a DNA molecule comprising the sequence of SEQ ID NO:1 or SEQ ID NO:3. In more preferred aspects of this embodiment the norleucine (and/or other non-standard amino acid) degrading protein is comprises a leucine hydrogenase having an amino acid sequence of SEQ ID NO:6, SEQ ID NO:8, SEQ ID NO:10, or SEQ ID NO:12; or a valine dehydrogenase having the amino acid sequence of SEQ ID NO:14; or a glutamate/leucine/phenylalanine/valine dehydrogenase having an amino acid sequence of SEQ ID NO:16. In the most preferred aspects of this embodiment the leucine dehydrogenase is encoded by a DNA molecule comprising the sequence of SEQ ID NO:5, SEQ ID NO:7, SEQ ID NO:9, or SEQ ID NO:11; or the valine dehydrogenase is encoded by a DNA molecule having the sequence of SEQ ID NO:13; or the glutamate/leucine/phenylalanine/valine dehydrogenase is encoded by a DNA molecule having the sequence of SEQ ID NO:15.

[0083] As indicated herein, various embodiments of the instant invention provide heterologous proteins and norleucine (and/or other non-standard amino acid) degrading protein (or fragments thereof) that are expressed from vectors transformed into a host cell (such as *E. coli*). In certain aspects of this embodiment, the heterologous protein and norleucine (and/or other non-standard amino acid) degrading protein are expressed from separate plasmids/vectors. In other embodiments they may be expressed from separate portions of the same plasmid or vector. Alternatively, one or both of the heterologous protein and norleucine (and/or other non-standard amino acid) degrading protein may be expressed from a site that is integral with the host cell's genome.

[0084] In any of the embodiments of the instant invention the expression of the heterologous protein and the co-expression or enhanced expression of the norleucine (and/or other non-standard amino acid) degrading protein may be expressed from either constitutive or from inducible promoters. Many constitutive and inducible promoters are well characterized and known to those skilled in the art.

[0085] According to various embodiments of the instant invention the methods are effective to reduce the percentage of heterologous protein containing norleucine (and/or one or more other non-standard amino acids) to below 5%. In more preferred aspects of this embodiment the percentage of heterologous proteins containing norleucine (and/or other non-standard amino

acid) is decreased to 4%, 3%, 2%, 1%, 0.5%, 0.25%, 0.01%, 0.05% and 0% or substantially 0% (meaning that the level of non-standard amino acids is below detectable limits).

[0086] Although it is not required, the present invention also provides for the simultaneous expression of a heterologous protein with two or more norleucine (and/or other non-standard amino acid) degrading proteins each of which has co-expression or enhanced expression. For example, bST can be simultaneously expressed with both wild-type and K92L variant *E. coli* GDH, if desired.

[0087] The instant invention also provides for a recombinant *E. coli* glutamate dehydrogenase protein wherein amino acid residue 92 has been changed from the native lysine to a leucine. In a particularly preferred embodiment the recombinant GDH protein comprises the sequence of SEQ ID NO:4. In an even more preferred embodiment, the GDH protein consists of or consists essentially of the sequence of SEQ ID NO:4.

[0088] Furthermore, if desired the instant invention provides for cells comprising the recombinant *E. coli* GDH comprising the K92L variant. In a preferred aspect of this embodiment the cells are *E. coli* cells. In an even more preferred embodiment, the cells are *E. coli* K-12 cells. Nevertheless, the instant invention is drawn to any cell containing the variant K92L GDH protein, such that it has an enhanced capacity to degrade norleucine.

[0089] The invention also provides for a recombinant DNA capable of encoding the K92L variant of the *E. coli* GDH protein (or catalytically active fragment thereof). A preferred aspect of this embodiment provides for a recombinant DNA molecule comprising the sequence provided as SEQ ID NO:3. Nevertheless one of skill in the art will appreciate that, owing to the degenerate nature of the genetic code, the recombinant DNA sequence may be varied without changing the sequence of the protein encoded thereby. Accordingly, various aspects of this embodiment of the instant invention are drawn to any sequence capable of encoding an *E. coli* K92L GDH variant.

[0090] Other aspects of this embodiment provide for recombinant DNA sequences encoding *E. coli* K92L GDH variants that further comprise variations at other amino acid residues. These variations are contemplated as being part of the instant invention so long as they do not reduce the ability of the encoded protein to degrade norleucine to a degree that makes it unsuitable for

use to prevent or substantially eliminate norleucine incorporation into a heterologous protein expressed in a cell.

[0091] Similarly various embodiments of the instant invention provide for norleucine (and/or other non-standard amino acid) degrading proteins that have been modified from their native primary structure (*e.g.*, the CS2 mutant of the phenylalanine dehydrogenase from *Thermoactinomyces intermedius* (Kataoka *et al.*, 1993)), but that still actively degrade non-standard amino acids, at rates that less than, equal to, or greater than the rates of the native protein.

[0092] Yet other aspects of this embodiment of the invention provide for DNA sequence encoding any of the proteins provided in the Examples, including, but not limited to leucine dehydrogenase from *Bacillus cereus, Bacillus subtilis, Nostoc sp.*, or *Shewanella oneidensis*; valine dehydrogenase from *Streptomyces avermitilis*, and glutamate/leucine/phenylalanine/valine dehydrogenase from *Nitrosomonas europaea.*

[0093] Other embodiments of the instant invention provide for a cell comprising any one or more of the recombinant DNA molecules described herein. In a preferred aspect of this embodiment, the cell is an *E. coli* cell. In an even more preferred embodiment the cell is an *E. coli* K-12 cell. Other embodiments provide for cells comprising any of the recombinant DNA molecules described herein wherein co-expression or enhanced expression of a norleucine degrading protein prevents or substantially eliminates incorporation of norleucine into a heterologous protein co-expressed in the cell.

[0094] Other embodiments of the instant invention provide for methods of producing a protein in and/or isolating a protein from a cell or microorganism. The various embodiments of these methods comprise the use of any combination of the cells, heterologous proteins, and norleucine (and/or other non-standard amino acid) degrading proteins described herein. The various aspects of this embodiment comprise co-expressing a heterologous protein and in a cell or microorganism with co-expression or enhanced expression of a norleucine (and/or other non-standard amino acid) degrading protein and then isolating protein from the microorganism. Preferably, the heterologous protein is isolated from the cell or microorganism. Methods for protein isolation are well known in the art and may be accomplished by means compatible with the selected heterologous protein.

[0095] Other aspects of this embodiment of the invention provide for methods comprising isolating proteins from a cell or microorganism that co-expresses a norleucine (and/or other non-standard amino acid) degrading protein and a heterologous protein.

EXAMPLES

[0096] The following examples are included to demonstrate preferred embodiments of the invention. It will be appreciated by those of skill in the art that the techniques disclosed in the following examples represent techniques discovered by the Applicant to function well in the practice of the invention, and thus can be considered to constitute preferred modes for its practice. However, those of skill in the art should, in light of the present disclosure, also appreciate that many changes can be made in the specific embodiments that are disclosed, while still obtaining a like or similar results, without departing from the invention. Thus, the examples are exemplary only and should not be construed to limit the invention in any way.

Example 1: Construction of plasmids co-expressing bovine somatotropin and either the wild-type or K92L variant glutamate dehydrogenases by *Escherichia coli*

[0097] When bovine somatotropin (bST) is expressed in *Escherichia coli*, the *E. coli* cell biosynthesizes norleucine and incorporates the norleucine into the bST protein and other cellular proteins (see, for example, Bogosian *et al.*, 1989). Thus, *E. coli* cells expressing bST protein are a good experimental system by which to test the effectiveness of norleucine degrading enzymes for reducing or eliminating norleucine incorporation into protein. The wild-type *E. coli* glutamate dehydrogenase (GDH) gene was cloned by polymerase chain reaction (PCR). A K92L variant of this GDH gene was also prepared by PCR. Both the wild-type and K92L variant glutamate dehydrogenase encoding genes were separately cloned into the bST expression vector pXT757. The construction and structural features of pXT757 are disclosed in WO 00/060103 and WO 02/051238 (which are each incorporated herein by reference). Briefly, the plasmid pXT757 is based on the well-known vector pBR322, and includes an inducible promoter driving the expression of the bovine somatotropin gene. Downstream of the bovine somatotropin gene is the constitutive lacUV5 promoter. The wild-type and K92L variant glutamate dehydrogenase genes were cloned into pXT757 downstream of the lacUV5 promoter so that the glutamate dehydrogenase proteins would be constitutively expressed (*i.e.* both before and after induction of

bovine somatotropin synthesis). In each plasmid, bST was expressed from an inducible promoter such as the cpex-20 promoter disclosed in WO 00/060103 and WO 02/051238). The new plasmid with the wild-type *E. coli* glutamate dehydrogenase gene was designated pXT814, and the new plasmid with the K92L variant of the *E. coli* glutamate dehydrogenase gene was designated pXT815.

[0098] Other plasmids co-expressing bovine somatotropin and a non-standard amino acid degrading protein were also constructed (see Table 3). These additional plasmids were prepared using methods analogous to those used to prepare pXT814 and pXT815, but the additional plasmids comprise different non-standard amino acid degrading proteins. It will be appreciated by those skilled in the art, that methods of preparing plasmids are well known. Moreover, it is well within the ability of the skilled artisan to prepare similar plasmids without undue experimentation.

Table 3 Additional co-expression plasmids

PLASMID	NON-STANDARD AMINO ACID DEGRADING PROTEIN	SOURCE OF GENE*
pXT1077	leucine dehydrogenase	*Bacillus cereus* (ATCC 14579)
pXT1078	leucine dehydrogenase	*Bacillus subtilis* (ATCC 6633)
pXT1079	leucine dehydrogenase	*Nostoc sp.* (ATCC 27893)
pXT1080	leucine dehydrogenase	*Shewanella oneidensis* (ATCC 700550)
pXT1081	valine dehydrogenase	*Streptomyces avermitilis* (ATCC 31267)
pXT1084	glutamate/leucine/phenylalanine/valine dehydrogenase	*Nitrosomonas europaea* (ATCC 19718)

*The ATCC number refers to the American-Type Culture Collection on-line catalog number for the species.

[0099] It will be appreciated by those skilled in the art that many other enzymes will likely also be effective for use according to the instant invention. Such enzymes may include members of a super-family of enzymes related to *E. coli* glutamate dehydrogenase and the proteins listed in Table 3. Such enzymes also include, but are not limited to, those enzymes listed in Table 4. At a minimum this protein super-family includes glutamate dehydrogenases, leucine dehydrogenases, valine dehydrogenases, phenylalanine dehydrogenases, glutamate/leucine/phenylalanine/valine dehydrogenases, and opine dehydrogenases.

Table 4 Proteins similar to *E. coli* glutamate dehydrogenase and the proteins of Table 3

Genbank Accession Number	Protein	Source Species
21222491	valine dehydrogenase	*Streptomyces coelicolor*
23100333	phenylalanine dehydrogenase	*Oceanobacillus iheyensis*
21402217	glutamate/leucine/phenylalanine/valine dehydrogenase	*Bacillus anthracis*
21399408	glutamate/leucine/phenylalanine/valine dehydrogenase	*Bacillus anthracis*
22778565	phenylalanine dehydrogenase	*Oceanobacillus iheyensis*
29607787	valine dehydrogenase	*Streptomyces avermitilis*
30249585	glutamate/leucine/phenylalanine/valine dehydrogenase	*Nitrosomonas europaea*
30138948	glutamate/leucine/phenylalanine/valine dehydrogenase	*Nitrosomonas europaea*
29830675	valine dehydrogenase	*Streptomyces avermitilis*
8928544	valine dehydrogenase	*Streptomyces coelicolor*
5918491	valine dehydrogenase	*Streptomyces coelicolor*
10172830	phenylalanine dehydrogenase	*Bacillus halodurans*
15612781	phenylalanine dehydrogenase	*Bacillus halodurans*
30022246	leucine dehydrogenase	*Bacillus cereus*
21402217	leucine dehydrogenase	*Bacillus anthracis*
34014423	leucine dehydrogenase	*Geobacillus stearothermophilus*
9087159	leucine dehydrogenase	*Bacillus licheniformis*
80215	leucine dehydrogenase	*Bacillus stearothermophilus*
1706414	leucine dehydrogenase	*Geobacillus stearothermophilus*
16079464	leucine dehydrogenase	*Bacillus subtilis*
15615328	leucine dehydrogenase	*Bacillus halodurans*
9087162	leucine dehydrogenase	*Thermoactinomyces intermedius*
1942796	leucine dehydrogenase	*Bacillus sphaericus*
23099324	leucine dehydrogenase	*Oceanobacillus iheyensis*
20808582	glutamate / leucine dehydrogenase	*Thermoanaerobacter tengcongensis*
20808583	glutamate / leucine dehydrogenase	*Thermoanaerobacter tengcongensis*
24374179	leucine dehydrogenase	*Shewanella oneidensis*
21242103	leucine dehydrogenase	*Xanthomonas axonopodis*
21230756	leucine dehydrogenase	*Xanthomonas campestris*
13272548	valine dehydrogenase	*Cytophaga sp.*
13516863	phenylalanine dehydrogenase	*Bacillus sp.*
17227922	leucine dehydrogenase	*Nostoc sp.*
23127785	glutamate / leucine dehydrogenase	*Nostoc punctiforme*
9087196	valine dehydrogenase	*Streptomyces cinnamonensis*

Genbank Accession Number	Protein	Source Species
9087194	valine dehydrogenase	*Streptomyces albus*
1174940	valine dehydrogenase	*Streptomyces ambofaciens*
731100	valine dehydrogenase	*Streptomyces fradiae*
25284773	phenylalanine dehydrogenase	*Bacillus halodurans*
2144245	phenylalanine dehydrogenase	*Bacillus badius*
2127513	valine dehydrogenase	*Streptomyces cinnamonensis*
2126840	phenylalanine dehydrogenase	*Bacillus sphaericus*
625925	phenylalanine dehydrogenase	*Rhodococcus sp*
538987	valine dehydrogenase	*Streptomyces coelicolor*
99040	phenylalanine dehydrogenase	*Thermoactinomyces intermedius*
3287880	opine dehydrogenase	*Arthrobacter sp.*
9087161	phenylalanine dehydrogenase	*Bacillus badius*
9087153	phenylalanine dehydrogenase	*Sporosarcina ureae*
118598	phenylalanine dehydrogenase	*Thermoactinomyces intermedius*
118597	phenylalanine dehydrogenase	*Bacillus sphaericus*
475596	phenylalanine dehydrogenase	*Rhodococcus sp*
13516863	phenylalanine dehydrogenase	*Bacillus sp*
13272548	valine dehydrogenase	*Cytophaga sp.*
10120619	phenylalanine dehydrogenase	*Rhodococcus sp.*
10120618	phenylalanine dehydrogenase	*Rhodococcus sp.*
10120617	phenylalanine dehydrogenase	*Rhodococcus sp.*
10120616	phenylalanine dehydrogenase	*Rhodococcus sp.*
295185	valine dehydrogenase	*Streptomyces coelicolor*
5107532	phenylalanine dehydrogenase	*Rhodococcus sp.*
5107531	phenylalanine dehydrogenase	*Rhodococcus sp.*
5107525	phenylalanine dehydrogenase	*Rhodococcus sp.*
5107524	phenylalanine dehydrogenase	*Rhodococcus sp.*
1228936	phenylalanine dehydrogenase	*Bacillus badius*
1147636	valine dehydrogenase	*Streptomyces cinnamonensis*
3126955	valine dehydrogenase	*Streptomyces albus*
216398	phenylalanine dehydrogenase	*Thermoactinomyces intermedius*
1842144	phenylalanine dehydrogenase	*Sporosarcina ureae*
499682	valine dehydrogenase	*Streptomyces ambofaciens*
532497	valine dehydrogenase	*Streptomyces fradiae*
529017	phenylalanine dehydrogenase	*Bacillus sphaericus*
16129715	glutamate dehydrogenase	*Escherichia coli*
26248016	glutamate dehydrogenase	*Escherichia coli*
15802172	glutamate dehydrogenase	*Escherichia coli*

Genbank Accession Number	Protein	Source Species
16764650	glutamate dehydrogenase	*Salmonella typhimurium*
16760596	glutamate dehydrogenase	*Salmonella enterica*
24112842	glutamate dehydrogenase	*Shigella flexneri*
45443083	glutamate dehydrogenase	*Yersinia pestis*
16124099	glutamate dehydrogenase	*Yersinia pestis*
37524148	glutamate dehydrogenase	*Photorhabdus luminescens*
15601908	glutamate dehydrogenase	*Pasteurella multocida*
23467136	glutamate dehydrogenase	*Haemophilus somnus*
46128953	glutamate dehydrogenase	*Haemophilus influenzae*
42630492	glutamate dehydrogenase	*Haemophilus influenzae*
16272153	glutamate dehydrogenase	*Haemophilus influenzae*
33603509	glutamate dehydrogenase	*Bordetella bronchiseptica*
33591596	glutamate dehydrogenase	*Bordetella pertussis*
48769923	glutamate dehydrogenase	*Ralstonia metallidurans*
30249585	glutamate dehydrogenase	*Nitrosomonas europaea*
46120572	glutamate dehydrogenase	*Methylobacillus flagellatus*
15806721	glutamate dehydrogenase	*Deinococcus radiodurans*
15599784	glutamate dehydrogenase	*Pseudomonas aeruginosa*
48728839	glutamate dehydrogenase	*Pseudomonas fluorescens*
26987411	glutamate dehydrogenase	*Pseudomonas putida*
15677557	glutamate dehydrogenase	*Neisseria meningitidis*
15794847	glutamate dehydrogenase	*Neisseria meningitidis*
29347380	glutamate dehydrogenase	*Bacteroides thetaiotaomicron*
33862896	glutamate dehydrogenase	*Prochlorococcus marinus*
15614664	glutamate dehydrogenase	*Bacillus halodurans*
18310500	glutamate dehydrogenase	*Clostridium perfringens*
48859402	glutamate dehydrogenase	*Clostridium thermocellum*
16262575	glutamate dehydrogenase	*Sinorhizobium meliloti*
29347383	glutamate dehydrogenase	*Bacteroides thetaiotaomicron*
50842991	glutamate dehydrogenase	*Propionibacterium acnes*
28377945	glutamate dehydrogenase	*Lactobacillus plantarum*
48849949	glutamate dehydrogenase	*Novosphingobium aromaticivorans*
48835833	glutamate dehydrogenase	*Thermobifida fusca*
23114323	glutamate dehydrogenase	*Desulfitobacterium hafniense*
25028538	glutamate dehydrogenase	*Corynebacterium efficiens*
21223063	glutamate dehydrogenase	*Streptomyces coelicolor*
38234122	glutamate dehydrogenase	*Corynebacterium diphtheriae*
24379360	glutamate dehydrogenase	*Streptococcus mutans*

Genbank Accession Number	Protein	Source Species
34540940	glutamate dehydrogenase	*Porphyromonas gingivalis*
16799644	glutamate dehydrogenase	*Listeria innocua*
16802603	glutamate dehydrogenase	*Listeria monocytogenes*
46906805	glutamate dehydrogenase	*Listeria monocytogenes*
23465213	glutamate dehydrogenase	*Bifidobacterium longum*
19553277	glutamate dehydrogenase	*Corynebacterium glutamicum*
50590027	glutamate dehydrogenase	*Streptococcus suis*
46205279	glutamate dehydrogenase	*Magnetospirillum magnetotacticum*
15645008	glutamate dehydrogenase	*Helicobacter pylori*
15903224	glutamate dehydrogenase	*Streptococcus pneumoniae*
15901165	glutamate dehydrogenase	*Streptococcus pneumoniae*
15612066	glutamate dehydrogenase	*Helicobacter pylori*
25011447	glutamate dehydrogenase	*Streptococcus agalactiae*
48845427	glutamate dehydrogenase	*Geobacter metallireducens*
22537482	glutamate dehydrogenase	*Streptococcus agalactiae*
29375982	glutamate dehydrogenase	*Enterococcus faecalis*
32266740	glutamate dehydrogenase	*Helicobacter hepaticus*
15894024	glutamate dehydrogenase	*Clostridium acetobutylicum*
48824795	glutamate dehydrogenase	*Enterococcus faecium*
39996407	glutamate dehydrogenase	*Geobacter sulfurreducens*
34558218	glutamate dehydrogenase	*Wolinella succinogenes*
48867880	glutamate dehydrogenase	*Haemophilus influenzae*
45515028	glutamate dehydrogenase	*Ralstonia eutropha*
46143225	glutamate dehydrogenase	*Actinobacillus pleuropneumoniae*
23129892	glutamate dehydrogenase	*Nostoc punctiforme*
19703823	glutamate dehydrogenase	*Fusobacterium nucleatum*
34764006	glutamate dehydrogenase	*Fusobacterium nucleatum*
46199513	glutamate dehydrogenase	*Thermus thermophilus*
37520702	glutamate dehydrogenase	*Gloeobacter violaceus*
15677330	glutamate dehydrogenase	*Neisseria meningitidis*
15794580	glutamate dehydrogenase	*Neisseria meningitidis*
17231747	glutamate dehydrogenase	*Nostoc sp.*
20807791	glutamate dehydrogenase	*Thermoanaerobacter tengcongensis*
28210980	glutamate dehydrogenase	*Clostridium tetani*
20807660	glutamate dehydrogenase	*Thermoanaerobacter tengcongensis*
42522302	glutamate dehydrogenase	*Bdellovibrio bacteriovorus*
46321123	glutamate dehydrogenase	*Burkholderia cepacia*
48767975	glutamate dehydrogenase	*Ralstonia metallidurans*

Genbank Accession Number	Protein	Source Species
46316063	glutamate dehydrogenase	*Burkholderia cepacia*
47573287	glutamate dehydrogenase	*Rubrivivax gelatinosus*
17545199	glutamate dehydrogenase	*Ralstonia solanacearum*
15643773	glutamate dehydrogenase	*Thermotoga maritima*
46132892	glutamate dehydrogenase	*Ralstonia eutropha*
21674833	glutamate dehydrogenase	*Chlorobium tepidum*
48785116	glutamate dehydrogenase	*Burkholderia fungorum*
15615281	glutamate dehydrogenase	*Bacillus halodurans*
15926547	glutamate dehydrogenase	*Staphylococcus aureus*
33592912	glutamate dehydrogenase	*Bordetella pertussis*
33596209	glutamate dehydrogenase	*Bordetella parapertussis*
23099265	glutamate dehydrogenase	*Oceanobacillus iheyensis*
16080831	glutamate dehydrogenase	*Bacillus subtilis*
16760686	glutamate dehydrogenase	*Salmonella enterica*
16765136	glutamate dehydrogenase	*Salmonella typhimurium*
42780691	glutamate dehydrogenase	*Bacillus cereus*
22974506	glutamate dehydrogenase	*Chloroflexus aurantiacus*
52143857	glutamate dehydrogenase	*Bacillus anthracis*
27467572	glutamate dehydrogenase	*Staphylococcus epidermidis*
42526508	glutamate dehydrogenase	*Treponema denticola*
46204709	glutamate dehydrogenase	*Magnetospirillum magnetotacticum*

Example 2: Co-expression of bovine somatotropin with norleucine (and other non-standard amino acid) degrading proteins

[00100] The plasmids pXT757, pXT814 pXT815, pXT1077, pXT1078, pXT1079, pXT1080, pXT1081, and pXT1084 were each separately transformed into the *E. coli* K-12 host strain LBB427 (LBB427 is a derivative of the common K-12 strain, W3110, differing only in that LBB427 has an *fhuA* gene knockout mutation). The conditions for the growth and induction of such bST expressing strains are disclosed in WO 00/060103. Briefly, the transformed strains were grown on minimal medium (*i.e.* no supplemental isoleucine, leucine, methionine, ALIMET®, rich medium supplement, or any other amino acid was added) at 37°C, from an initial OD_{550} of 0.3. When the OD_{550} reached 0.8., the cultures were induced by the addition of nalidixic acid to a final concentration of 50 micrograms per ml. The bovine somatotropin protein was isolated and analyzed for norleucine content. The assay for the norleucine content of bovine

somatotropin is described in detail in Bogosian *et al.*, 1989. Briefly, the assay employs a high performance liquid chromatographic (HPLC) column run under conditions that resolve norleucine-free bovine somatotropin and norleucine-containing bovine somatotropin into separate peaks, which can then easily be quantified. The norleucine-containing bovine somatotropin was separated from the bulk of bovine somatotropin with a Perkin-Elmer Series 4 HPLC using a Vydac C-18 column. The chromatographic conditions were a flow rate of 2 ml/minute with constant 40 mM trifluoroacetic acid, followed by a gradient of 54-60% acetonitrile over 24 minutes, followed by a gradient of 60-75% acetonitrile over 6 minutes. The strain transformed with pXT757 was used as a control (*i.e.* one not co-expressing any norleucine (or other non-standard amino acid) degrading enzyme. The resulting percentages of bST containing norleucine were as shown in Table 5.

Table 5 Reduction or elimination of norleucine from protein

Host Strain (Plasmid)	Description	Percent of protein containing norleucine
LBB427 (pXT757)	control, no co-expressed norleucine degrading protein	17.4
LBB427 (pXT814)	co-expression with wild-type GDH	0.9
LBB427 (pXT815)	co-expression with K92L variant GDH	0.6
LBB427 (pXT1077)	co-expression with leucine dehydrogenase	below detection limit of 0.03
LBB427 (pXT1078)	co-expression with leucine dehydrogenase	0.55
LBB427 (pXT1079)	co-expression with leucine dehydrogenase	0.57
LBB427 (pXT1080)	co-expression with leucine dehydrogenase	below detection limit of 0.03
LBB427 (pXT1081)	co-expression with valine dehydrogenase	1.14
LBB427 (pXT1084)	co-expression with glutamate/ leucine/phenylalanine/valine dehydrogenase	below detection limit of 0.03

[00101] As the data presented in Table 5 demonstrate, the cloned *E. coli* wild-type glutamate dehydrogenase gene product degrades much of the norleucine, thereby reducing the incorporation of norleucine from 17.4% to 0.9%. The K92L variant glutamate dehydrogenase

gene product even more effectively reduces the percentage of proteins containing norleucine, to a level of 0.6%. Similarly, the leucine dehydrogenase gene products from *Bacillus subtilis* and *Nostoc* sp., and the valine dehydrogenase gene product from *Streptomyces avermitilis*, also effectively reduce the percentage of proteins containing norleucine. Moreover, the leucine dehydrogenase gene products from *Bacillus cereus* (ATCC 14579), *Shewanella oneidensis* (ATCC 700550) and the glutamate/leucine/phenylalanine/valine dehydrogenase gene product from *Nitrosomonas europaea* (ATCC 19718) each reduce the percentage of protein containing norleucine to substantially zero (*i.e.*, below detectable limits).

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[00102] The following references, to the extent that they provide exemplary procedural or other details supplementary to those set forth herein, are specifically incorporated herein by reference.

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